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THE EFFICACY OF VARIOUS THIOLS AS ANTIDOTES TO LEWISITE[1]

BY S. D. SIMPSON[2] AND L. YOUNG[3]

Abstract

The following thiols were tested for their effectiveness in counteracting the systemic toxic action of lewisite (β-chlorovinyldichloroarsine) in the rat: 1- and 2-mercaptopropane, 2-mercaptoethanol, 1,2-dimercaptoethane, 1,2- and 1,3-dimercaptopropane, 1,2,3-trimercaptopropane, 1,2-dimercapto-*n*-butane, 2,3-dimercaptopropanol, 1,3-dimercapto-2-propanol, 2,2′-dimercaptodiethyl ether, 2,2′-dimercaptodiisopropyl ether, and 3,3′-dimercaptodipropyl ether. The toxicity and volatility of these compounds were also studied.

The tests of antidotal activity were conducted by applying the thiol to the skin at a site which had been dosed with approximately twice the LD_{50} of lewisite. Under these conditions the monothiols gave no evidence of antidotal activity whereas all the dithiols and the trithiol studied showed some activity. 2,3-Dimercaptopropanol (BAL) and its isomer, 1,3-dimercapto-2-propanol, were much more effective as antidotes to lewisite than any of the other compounds tested. Not only did 2,3-dimercaptopropanol show a somewhat higher antidotal activity than its isomer, but it was also found to be much less toxic.

Introduction

The view that the toxic action of trivalent arsenical compounds is related to their reaction with essential thiol compounds in the organism was advanced by Voegtlin, Dyer, and Leonard (11) in 1923. These workers also found that under suitable conditions certain monothiols can diminish or prevent the toxic action of arsenical compounds (12) and this finding was supported by results obtained in other investigations (1, 4). The outbreak of World War II provided a stimulus to the search for antidotes to arsenical compounds, and in particular to chemical warfare agents such as lewisite (β-chlorovinyldichloroarsine). In 1940, Peters, Stocken, and Thompson, in the course of a study of the interaction of lewisite and the thiol groups of kerateine, found that most of the arsenic in the derived protein was combined with two thiol groups and they were led to the conclusion that "simple dithiol compounds might form relatively stable ring compounds with lewisite and other trivalent arsenical compounds and so compete successfully with 'dithiol' proteins in the tissues" (3). This, in turn, led them to the important finding that the dithiol, 2,3-dimercaptopropanol (later named BAL, British Anti-Lewisite, in the United States) is highly effective in preventing the local and systemic

[1] *Manuscript received February 4, 1950.*
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actions of lewisite. Reviews of work on BAL have been published by Peters, Stocken, and Thompson (3), Peters (2), Thompson (10), and Stocken and Thompson (8). Work on BAL in the United States has been described by Waters and Stock (13) and Sulzberger and Bair (9), and Canadian researches on BAL have been reviewed by Young (15).

The work described herein was carried out in 1942-43 (7) following the receipt of early accounts of the researches of the Oxford workers. It was undertaken in order to compare the toxicity of BAL and its antidotal activity in lewisite poisoning with those of a series of related compounds. For this purpose the following thiols were synthesized by Simpson (5, 6): 1,2-dimercaptoethane, 1,2-dimercaptopropane, 1,3-dimercaptopropane, 1,2,3-trimercaptopropane, 1,2-dimercapto-*n*-butane, 1,3-dimercapto-2-propanol, 2,2′-dimercaptodiethyl ether, 2,2′-dimercaptodiisopropyl ether, and 3,3′-dimercaptodipropyl ether. These compounds, together with 1- and 2-mercaptopropane, 2-mercaptoethanol, and 2,3-dimercaptopropanol, were tested for their toxicity and their activity as antidotes to lewisite. As the main method of administration of an antilewisite compound in the field appeared likely to be by direct application to the site of contamination, the tests of toxicity and antidotal activity were conducted by applying the thiols to the skin. Among the factors which influenced the results obtained under these conditions was the volatility of the compounds being tested, and for this reason all the animal tests were performed in a room maintained at 25 ± 1° C. In order that the volatility of the thiols might be taken into consideration in the interpretation of the results of the tests of antidotal activity, a quantitative study was made of the evaporation of the thiols at 25° and 37° C.

Materials and Methods

The 1- and 2-mercaptopropane and the 2-mercaptoethanol used were products of the Eastman Kodak Company, the 2,3-dimercaptopropanol was provided by the Directorate of Chemical Warfare, Department of National Defence, Ottawa, and the remaining nine thiols were synthesized by Simpson (5, 6). The sample of lewisite used had been carefully purified and was colorless (b.p. 79.5-80.5° at 14 mm. pressure; $n_D^{20} = 1.6091$; $d_4^{25} = 1.883$). It was kindly provided by Dr. G. F Wright, Department of Chemistry, University of Toronto.

White rats, all of the same strain, from the colony of the Department of Biochemistry, University of Toronto, were used in the animal experiments. Equal numbers of male and female rats were used under each set of conditions studied. Each rat weighed between 150 and 185 gm. In every test the site of application of the agent or agents was the skin of the mid-lumbar region. Before the animal was dosed it was anesthetized with ether and the hair in the lumbar region was cut as closely to the skin as possible by means of electric shears and scissors. The temperature of the room in which the dosing experiments were performed was maintained at 25 ± 1° C. At the end of the dosing operation each rat was placed in a separate metal cage in which it was

provided with food (Master Fox Breeding Ration—Toronto Elevators Limited) and water *ad libitum*. The animal was then weighed daily for a week and if it was gaining weight at the end of this period it was classed as having survived. If it was losing weight at this time it was kept under observation for a further period to determine whether it would recover.

In order to determine the toxicity of a thiol the compound was measured on to the skin of the anesthetized animals from a pipette with 0.01 ml. graduations, and was rubbed into the skin for five minutes with the flattened end of a glass rod. Anesthesia was continued for 30 min. after dosing. The toxicity of lewisite was determined under similar conditions, except that this agent was applied by means of a micropipette of the type described by Young (14, 16) and was not rubbed into the skin. In testing the antidotal activity of a thiol, each rat in the test group was anesthetized with ether and was dosed on the skin with 40 mgm. of lewisite per kgm. body weight (approximately 2 × LD_{50}—see below). After a measured interval of time, 0.05 ml. of the thiol was applied to the contaminated area and was rubbed into the skin for five minutes. In those experiments in which the interval between the application of the lewisite and the thiol was 15 or 30 min. the animals were kept anesthetized until after the thiol had been applied. When the interval was one hour or longer, the animals were allowed to regain consciousness 30 to 45 min. after they had been dosed with lewisite and were anesthetized again before they were treated with the thiol.

The procedure used for measuring the evaporation of a thiol was as follows. By means of a pipette, 0.16 ml. of the thiol was measured on to a microscope coverslip supported on a watch glass and its weight was determined immediately. The amounts of thiol which evaporated in 15, 30, and 60 min. were then determined by weighing. The evaporation at 25° C. was measured in a room maintained at that temperature. All the weighings were made on an oil-damped balance and each weighing was completed in less than one minute. The measurements at 37° C. were made by placing the microscope coverslip carrying the thiol in a large incubator for 15, 30, or 60 min., and then weighing it on the oil-damped balance within one to two minutes after removal from the incubator. All determinations were made in duplicate.

Results

Toxicity of the Thiols

The results obtained in studies of the toxicity of the thiols are shown in Table I. In many experiments the administration of the thiol gave rise to profuse lachrymation. Most of the rats which received lethal doses of thiol died within 24 hr. after dosing, and death was often preceded by violent convulsions. Some of the animals died several days after being dosed, and during this period they lost weight and at the time of death they were very emaciated. The rats which received sublethal doses of thiol usually lost weight for a few days and then rapidly gained in weight. Those rats which were

TABLE I

THE TOXICITY OF THIOLS WHEN APPLIED TO THE SKIN OF THE RAT

Thiol	mM. of thiol per ml.	Dose of thiol applied (ml./kgm. body wt.)						
		0.3	0.6	0.9	1.2	1.8	2.4	3.0
		Rats surviving in each group of four rats						
1-Mercaptopropane	11.0	–	–	–	–	–	–	4
2-Mercaptopropane	10.6	–	–	–	–	–	–	4
2-Mercaptoethanol	14.4	–	4	3	2	3	0	1
1,2-Dimercaptoethane	11.9	–	–	4	3	0	–	–
1,2-Dimercaptopropane	9.8	–	4	3	3	3	1	1
1,3-Dimercaptopropane	10.0	–	–	–	4	3	2	0
1,2,3-Trimercaptopropane	8.8	3	1	0	–	–	–	–
1,2-Dimercapto-*n*-butane	8.5	–	4	3	2	0	–	–
2,3-Dimercaptopropanol	10.0	–	4	4	4	3	0	0
1,3-Dimercapto-2-propanol	10.0	1	0	–	–	–	–	–
2,2′-Dimercaptodiethyl ether	8.1	–	4	2	1	0	–	–
2,2′-Dimercaptodiisopropyl ether	6.3	–	–	4	2	0	–	–
3,3′-Dimercaptodipropyl ether	6.3	–	–	4	2	0	–	–

dosed with amounts of thiol approaching the lethal level showed the greatest loss of weight. For several days after being dosed some animals were hyper-excitable when handled.

Toxicity of Lewisite

The data obtained in the determination of the toxicity of lewisite when applied to the skin of the rat are shown in Table II. The animals which

TABLE II

THE TOXICITY OF LEWISITE WHEN APPLIED TO THE SKIN OF THE RAT

No. of rats in group	Av. wt. of rats in group, gm.	Mgm. of lewisite applied	Dose of lewisite mgm./kgm. body wt.	Per cent mortality
10	170	1.0	6	0
10	170	2.0	12	0
10	168	3.0	18	30
10	173	4.0	23	90
10	169	5.0	30	100

received lethal doses of lewisite usually developed pleural effusion and severe diarrhoea, and they rarely survived for longer than 24 hr. after being dosed. From the data in Table II it appears that the LD_{50} of the sample of lewisite used in the present work was between 18 and 23 mgm. per kgm. body weight.

Antidotal Activity of the Thiols

The results obtained in studies of the efficacy of the thiols as antidotes to lewisite are shown in Table III.

TABLE III

THE ANTIDOTAL ACTIVITY OF THIOLS APPLIED CUTANEOUSLY TO RATS DOSED WITH 40 MGM. LEWISITE PER KGM. (APPROX. 2 × LD_{50})

Thiol	mM. of thiol applied (0.05 ml.)	Interval in minutes between application of lewisite and application of thiol						
		15	30	60	90	120	180	240
		Rats surviving in each group of four rats						
1-Mercaptopropane	0.55	0	–	–	–	–	–	–
2-Mercaptopropane	0.53	0	–	–	–	–	–	–
2-Mercaptoethanol	0.72	0	–	–	–	–	–	–
1,2-Dimercaptoethane	0.60	4	2	0	–	–	–	–
1,2-Dimercaptopropane	0.49	3	1	0	–	–	–	–
1,3-Dimercaptopropane	0.50	4	3	1	–	–	–	–
1,2,3-Trimercaptopropane	0.44	4	4	1	–	–	–	–
1,2-Dimercapto-*n*-butane	0.43	2	1	0	–	–	–	–
2,3-Dimercaptopropanol	0.50	4	4	4	4	3	3	1
1,3-Dimercapto-2-propanol	0.50	3	4	4	4	4	0	–
2,2′-Dimercaptodiethyl ether	0.41	4	4	2	–	–	–	–
2,2′-Dimercaptodiisopropyl ether	0.32	4	4	0	–	–	–	–
3,3′-Dimercaptodipropyl ether	0.32	4	4	1	–	–	–	–

Each animal was dosed with 40 mgm. of lewisite per kgm. body weight. This was approximately twice the LD_{50}, and every rat in a control group of 16 animals died within 24 hr. of being dosed with this amount of the agent. The amount of thiol used in each test of antidotal activity was 0.05 ml., i.e. approximately 0.30 ml. per kgm. weight. This was a sublethal dose for every thiol tested except 1,2,3-trimercaptopropane and the highly toxic dithiol, 1,3-dimercapto-2-propanol. It appeared, however, that the use in all experiments of a volume of thiol which corresponded to a sublethal dose of 1,3-dimercapto-2-propanol would have necessitated reducing the amount to a level which would have been unsatisfactory for testing the antidotal activity of many of the thiols. It is interesting to note that there were 19 survivors out of 20 rats dosed with lewisite and treated at intervals up to two hours with 1,3-dimercapto-2-propanol (see Table III), a finding which suggests that the level of this thiol which can be tolerated is increased in animals dosed with lewisite. Those rats which died after being dosed with lewisite and treated with a thiol usually survived no longer than 24 hr. after being dosed. The animals which recovered usually lost weight for several days after being dosed and thereafter they gained in weight rather slowly.

The most effective antidotes were those thiols which evaporated slowly, and the following control experiment was carried out in order to find whether protection against lewisite was afforded by the mere application of a liquid with low volatility to the site of contamination. The agent selected was glycerol, because of its physical characteristics, its lack of toxicity, and its chemical relationship to the most effective antidotes tested, namely the dimercaptopropanols. Eight rats were dosed with 40 mgm. of lewisite per

kgm. body weight and 15 min. later 0.05 ml. of glycerol was applied to the site of contamination on four of the animals and was rubbed into the skin for five minutes. The remaining four rats were treated with glycerol in a similar manner 30 min. after being dosed with lewisite. All eight rats died within 24 hr. after being dosed.

Evaporation of the Thiols

The purpose of the experiments in which the evaporation of the thiols was studied was merely to obtain evidence of any gross differences in the volatilities of the thiols.

The evaporation of the thiols was measured at 25° and 37° C. because these were the extreme temperatures, i.e. room temperature and body temperature of the rat, to which the thiols could possibly have been subjected during the animal experiments. From the results obtained (see Table IV) it appears

TABLE IV

THE EVAPORATION OF THIOLS IN STILL AIR AT 25° AND 37° C.

Thiol	25° C. Per cent (by wt.) of thiol which evaporated in:			37° C. Per cent (by wt.) of thiol which evaporated in:		
	15 min.	30 min.	60 min.	15 min.	30 min.	60 min.
1-Mercaptopropane	(100)	—	—	(100)	—	—
2-Mercaptopropane	(100)	—	—	(100)	—	—
2-Mercaptoethanol	8	17	36	17	32	59
1,2-Dimercaptoethane	28	61	(100)	58	(100)	—
1,2-Dimercaptopropane	49	91	(100)	64	(100)	—
1,3-Dimercaptopropane	11	23	46	24	45	88
1,2,3-Trimercaptopropane	1	2	4	2	4	7
1,2-Dimercapto-*n*-butane	28	59	96	48	87	(100)
2,3-Dimercaptopropanol	<1	<1	<1	1	2	3
1,3-Dimercapto-2-propanol	<1	<1	1	<1	1	2
2,2′-Dimercaptodiethyl ether	1	2	4	3	6	11
2,2′-Dimercaptodiisopropyl ether	2	4	8	3	6	12
3,3′-Dimercaptodipropyl ether	<1	<1	1	<1	1	2

that there were great differences in the rates at which the compounds evaporated. The possible significance of the volatility of certain of the thiols in relation to their effectiveness when applied cutaneously as antidotes to lewisite is discussed below.

Discussion

The main conclusion which can be drawn from the present work is that 2,3-dimercaptopropanol (BAL), under the test conditions used, was more effective in preventing the systemic toxic action of lewisite than any of the other thiols studied. These compounds consisted of three monothiols, eight dithiols, and one trithiol.

The only thiol found to have an antidotal activity approaching that of 2,3-dimercaptopropanol was its isomer, 1,3-dimercapto-2-propanol. Both of these compounds proved to be effective antidotes even when their administration was delayed for two hours after contaminating rats with amounts of lewisite which would otherwise have caused death. These two compounds, however, showed a marked difference in toxicity. Not only was the 1,3-compound found to be much more toxic than its isomer, but it was also more toxic than any other thiol tested.

The monothiols, 1- and 2-mercaptopropane, were found to have no value as antidotes to lewisite under the test conditions used. These thiols were the most volatile of the compounds studied, and their lack of antidotal activity may possibly have been related to their rapid evaporation from the skin. It is noteworthy, however, that 2-mercaptoethanol, the other monothiol studied, showed low volatility but gave no evidence of antidotal activity.

All the dithiols and the trithiol studied in the present work showed some degree of antidotal activity. Much higher activity was observed with 2,3-dimercaptopropanol and 1,3-dimercapto-2-propanol than with any of the other 1,2- and 1,3-dithiols studied, namely 1,2-dimercaptoethane, 1,2- and 1,3-dimercaptopropane and 1,2-dimercapto-*n*-butane. These latter compounds were found to be much more volatile than the dimercaptopropanols, and this may well have been one of the factors which gave rise to the observed differences in activity. An indication that the increase in antidotal activity brought about by the introduction of a hydroxyl group into 1,2- or 1,3-dimercaptopropane is not wholly related to a decrease in volatility is provided by a comparison of the activity and volatility of the dimercaptopropanols with those of 1,2,3-trimercaptopropane. The volatility of this latter compound was found to be close to that of the dimercaptopropanols, but as an antidote to lewisite it proved to be little better than 1,3-dimercaptopropane.

If the efficacy of a dithiol as an antidote to an arsenic compound is related to its ability to form a cyclic dithioarsinite, then the stability of the ring compound becomes a factor in determining the antidotal activity of the thiol (3). The stability of the ring compound is itself influenced by the nature and number of the constituents of the cyclic structure. Some interest attaches, therefore, to the antidotal behavior of the 1,5-dithiols, 2,2′-dimercaptodiethyl ether and 2,2′-dimercaptodiisopropyl ether, and the 1,7-dithiol, 3,3′-dimercaptodipropyl ether, for if these compounds react with trivalent arsenicals to form cyclic derivatives, the 1,5-dithiols give rise to eight-membered ring compounds, and the 1,7-dithiol forms a 10-membered ring compound. The dithiol ethers showed low volatility and when applied to the skin they appeared to be somewhat more effective as antidotes than those 1,2- and 1,3-dithiols which were rather volatile. On the other hand, they were much less active than the dimercaptopropanols. If there was formation of 8- and 10-membered ring compounds of the type described above, these may have been much less stable than the five- and six-membered rings formed by the interaction of lewisite and dimercaptopropanols. It must be borne

in mind, however, that even though formation of a stable cyclic dithioarsinite may favor the antidotal activity of a dithiol, such factors as the distribution, metabolism, excretion, and toxicity of the thiol and those of any derivatives which it forms with the arsenic compound may also greatly influence the outcome of its use as an antidote in arsenic poisoning.

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THYROID FUNCTION IN ESSENTIAL HYPERTENSION[1]

By A. E. Thompson, Nancy E. Mathers, and W. F. Perry

Abstract

The functional condition of the thyroid glands of 32 hypertensive subjects was assessed by measurements of the plasma protein bound iodine. These did not differ significantly from those of a control group. No correlation was found within the hypertensive group between the severity of the disorder and the level of the plasma protein bound iodine. It was concluded that the activity of the thyroid gland is not affected by the presence of hypertension.

Introduction

Investigations of the state of the thyroid gland in hypertension have chiefly depended on estimations of the basal metabolic rate. There seems to be general agreement that hypertensive subjects show a higher than normal incidence of elevated rates but as recently as 1947 Rosenkrantz and Marshall (7) considered that it was a matter of opinion whether this was due to hyperactivity of the thyroid gland or merely a reflection of increased cardiac work or complications such as congestive heart failure. Measurements of the total blood iodine have not clarified the situation as Turner *et al.* (10) found it low while Curtis and Fertman (1) found a tendency to elevation.

Plasma protein bound iodine values have been shown (3, 4) to bear a close relationship to the clinical status of patients with disordered thyroid function. It also appears that the values are not disturbed by extra thyroidal disorders except in the presence of hypoproteinemia (5), obesity (11), or medication with organic iodine compounds (3). Using this measurement Salter *et al.* (8) found normal values in four cases of hypertension but Perry and Cosgrove (4) reported elevated values in two of six cases. The present study was undertaken to reassess and extend these observations.

Subjects and Methods

The subjects studied are listed in Table I, and were selected chiefly from the Outpatients' Department without regard to the degree of hypertension, its duration, or the complications. A special effort was made to include males while cases of clinically obvious thyroid dysfunction or obesity were excluded. The subjects include five of the six cases reported by Perry and Cosgrove. The sixth was a woman of 68 who on repeated examination had shown elevation of the plasma protein bound iodine. Following the appearance of their report, death occurred due to rupture of a myocardial infarct and at autopsy the thyroid gland was profoundly atrophic. Because of this and because of a history of prolonged medication with a variety of compounds which may have

[1] *Manuscript received April 1, 1950.*

Contribution from the Department of Medicine and the Department of Physiology and Medical Research, University of Manitoba, and from the Winnipeg General Hospital, Winnipeg, Canada.

contained iodine it was decided that she could no longer be considered a simple case of hypertension. The other case stated to have had a high plasma protein bound iodine value has been reinvestigated and is reported here as case No. 5. The previous value of 14.0 μgm.% must be ascribed to a technical error.

The blood pressure was measured by the standard auscultatory procedure. Each resting pressure recorded in Table I was the mean of seven measurements made over a 45 min. period after 20 min. rest in the recumbent position, while the maximal pressure was the highest noted in the patient's chart. Gross enlargement of the heart was considered to be present when the apex beat was more than 10 cm. from the midsternal line or when a radiogram showed that its transverse diameter exceeded one-half the internal breadth of the chest.

Left ventricular strain was diagnosed by the standard electrocardiographic criteria. The severity of the changes in the ocular fundus were assessed according to the grading of Keith *et al.* (2). The basal metabolic rate was measured using the Benedict–Roth closed circuit equipment.

The plasma protein bound iodine was measured in duplicate on venous blood by the method of Perry and Cosgrove (4). In each case the total plasma protein measured by the method of Phillips *et al.* (6) was found to be within the normal range.

Results

The mean protein bound plasma iodine of the 32 hypertensive subjects was 4.9 μgm. ± 1.5 (S.D.) with a range of 1.9 to 9.0 μgm.%. In a previously reported (4) group of 34 normal subjects the mean was 5.9 μgm.% ± 1.3 (S.D.) with a range of 4.0 to 9.3 μgm.%. An analysis of variance (9) showed the difference between the groups to be of slight significance, $P > .01$ and $< .05$.

The pertinent laboratory findings and clinical details of the hypertensive subjects are listed in Table I in ascending order of their plasma protein bound iodine concentrations. It will be seen that the subjects with the lowest values are similar to those with highest values in regard to resting blood pressure, maximal blood pressure, cardiac enlargement, electrocardiographic findings, ocular changes and symptoms. Thus it is clear that in the present series the protein bound iodine values cannot be correlated with any of the various criteria considered to reflect the severity or chronicity of the hypertensive condition.

Discussion

The results reported here indicate that the plasma protein bound iodine values of the hypertensive subjects were similar or possibly slightly lower than those of healthy individuals. Such differences as were found may be due to the fact that the values for healthy individuals were obtained largely on males under the age of 35, whereas almost all the hypertensive subjects

TABLE I

DETAILS OF HYPERTENSIVE SUBJECTS

No.	Sex	Age	Protein bound iodine, μgm.%	Blood pressure				B. M. R.	Cardiac exam.*		Fundus**	Symptoms (duration in years)			
				Resting		Maximal									
				Sys.	Dia.	Sys.	Dia.		Size	Strain		Headache	Dyspnea	Angina	Failure***
1	M	55	1.9	200	130	240	190		0	+	2	0	0	0	0
2	M	54	1.9	200	120	220	155	−10	+	+	2	4	1	0	0
3	M	85	3.4	190	110	210	125	0	+			0	5	0	0
4	F	47	3.6	180	115	250	165	+10	+	+	3	0	7	0	6 (4)
5	F	60	3.6	195	80	220	110	0	+	+	1	0	2	0	1 (1)
6	M	45	3.6	180	120	240	140		+	0	2	2	0	0	0
7	M	59	3.7	240	140	275	155		0	+	1	0	0	0	0
8	F	65	3.8	200	115	210	125	+ 8	+	+	3	0	0	4	0
9	F	45	4.1	220	130	250	140	0	+	0	1	0	4	0	0
10	F	23	4.2	165	95	180	105	+ 8	0	0	0	0	0	0	0
11	F	51	4.2	200	115	240	140	+ 1	+		3	1	6	0	0
12	M	73	4.2	180	100	210	120		0	+	1	0	10	0	0
13	F	49	4.4	240	145	260	160	− 5	0	0		9	8	0	0
14	F	46	4.4	180	100	230	110	−12	0	0	2	2	2	0	0
15	F	58	4.4	180	105	230	120	− 2	+	0	3	0	4	0	4 (2)
16	F	68	4.4	185	100	205	140	− 3	+	+	2	8	0	3	0
17	M	56	4.5	180	115	200	145	+18	0	+	2	6	0	0	0
18	F	48	4.7	200	120	245	140	+10	0		1	8	0	0	0
19	M	45	4.7	170	110	175	120	+ 6	0	0	0	0	0	0	0
20	M	59	4.8	175	100	190	110		0	0	1	4	0	0	0
21	M	31	4.9	200	130	250	170	+15	+	+	4	1	0	0	0
22	F	56	5.0	170	100	240	120	− 8	+	+	2	0	6	5	0
23	F	56	5.1	160	115	250	150	+10	+	+		0	1	0	1 (1)
24	M	58	5.5	180	105	190	110		+	+	1	1	1	0	0
25	F	57	5.7	130	85	240	150	+13	+	+	3	0	10	10	10 (1)
26	F	64	5.8	180	80	250	110	0	+	+	1	0	10	10	1 (1)
27	M	53	6.3	160	125	240	145	+ 5	+	+	1	0	1	0	1 (1)
28	F	62	6.4	180	95	190	120	− 9	0	0	2	0	7	10	6 (1)
29	F	22	6.9	160	115	260	190	− 4	0	0	1	0	0	0	0
30	F	33	7.6	160	120	170	130	− 2	0	0	1	4	5	0	0
31	F	47	7.7	220	120	280	155	+11	0	0	2	7	0	0	0
32	M	48	9.0	190	100	260	165	+11	0	0	4	1	1	0	0

* *Presence of gross cardiac enlargement and electrocardiographic evidence of left ventricular strain is indicated by a + sign.*

** *Grade of hypertensive retinopathy.*

*** *Figure in brackets indicates number of episodes of congestive heart failure.*

Note: Cases 1, 23, and 25 had a thyroidectomy five or more years previously. Cases 4, 5, 23, 24, 25, 26, and 27 were receiving digitalis.

were well beyond this age and females outnumbered the males. Furthermore, within the hypertensive group the severity of the condition bore no relation to the level of protein bound iodine. Hence it is concluded that the activity of the thyroid gland is not affected by the presence of essential hypertension.

Acknowledgments

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THE EFFECT OF ADRENOCORTICOTROPHIC HORMONE AND SURGICAL OPERATIONS ON IODINE EXCRETION[1]

By J. P. Gemmell and W. F. Perry

Abstract

Further investigations have been carried out on the previously reported increase in iodine excretion which occurs following the stress of surgical operations. By means of adrenocorticotrophic hormone it was demonstrated in four subjects that increased activity of the adrenal cortex does not of itself cause an increase in iodine excretion. In six patients subjected to elective surgical procedures it was shown with the aid of radioactive iodine that the inorganic iodide of the body tissues is not the source of increased iodine excretion which follows such operations. Other possible causes and sources of the postoperative increase in iodine excretion are discussed.

Introduction

In a previous communication (3) it was demonstrated that the stress of surgical operations in healthy human subjects, besides being associated with increased adrenal cortical activity, indicated by elevated urinary excretion of corticosteroids, was also accompanied by a very marked and sudden increase in the excretion of iodine in the urine.

Whether this alteration in iodine metabolism was a result of the extra production of cortical hormones or alternatively was an unassociated stress phenomenon was not established. In addition, although several possible sources of the extra urinary iodine were postulated its actual origin remained unknown.

The purpose of the present communication is to report observations concerning these points.

Subjects

The subjects of these investigations are detailed in Table I. As in the previous series, all those on whom surgery was performed were essentially normal healthy people who were hospitalized for elective surgical procedures. On the basis of the usual clinical and laboratory criteria, none of the subjects were considered to have any thyroid or adrenal dysfunction.

Methods

The analytical procedures for urinary constituents have been previously described (3). In order to examine the effect of increased adrenal cortical activity on iodine excretion in the absence of operative trauma, the adrenal cortex was stimulated by means of its trophic hormone (ACTH-Armour). This was administered intramuscularly to four patients in 25 mgm. amounts three to four times daily for from two and a half to five days.

[1] *Manuscript received March 13, 1950.*

Contribution from the Department of Medicine and the Department of Physiology and Medical Research of The University of Manitoba and from the Winnipeg General Hospital, Winnipeg, Canada.

TABLE I

SUBJECTS OF EXPERIMENTS

Subject	Age	Sex	Diagnosis or operative procedure
1	45	M	Normal
2	43	M	Dermatitis
3	28	M	Disseminated sclerosis
4	42	M	Rheumatoid arthritis
5	41	M	Plate to radius and ulna
6	22	M	Excision of metatarsals
7	47	M	Arthrodesis; foot
8	25	M	Herniotomy
9	27	M	Herniotomy
10	56	M	Herniotomy
11	21	M	Normal
12	24	M	Normal
13	39	M	Normal
14	20	M	Normal

Concerning the origin of extra iodine excreted postoperatively it was decided to investigate the inorganic iodine of the body tissues as a possible source. This objective was attained by means of the following procedure.

A tracer dose of radioactive iodine as NaI^{131} was administered and two hours allowed for diffusion throughout the body fluids in order to label the inorganic iodide of the tissues with radioiodine. The ratio of the concentration of isotopic iodine (% dose per 100 ml.) to that of stable iodine (μgm. per 100 ml.) was then determined in either plasma or urine or both. This ratio was determined before submitting subjects to operation and two to four hours after operation, at which time it has been observed that the plasma and urine levels of iodine are elevated. If the source of the postoperative iodine increase were from the labelled source, i.e., the inorganic body iodine, there should be no great change in the ratio following operation. On the other hand, if the extra iodine were from nonlabelled sources there should be a marked alteration of the ratio.

This measurement was performed in six patients who were subjected to operative trauma, five of whom showed postoperative increases in iodine excretion, and in four other persons who were not subjected to operation.

Results

The results of ACTH administration in four subjects are shown in Fig. 1 together with, for comparison, the mean results reported for surgical trauma (3). It will be seen that increased levels of adrenal cortical hormones do not in themselves lead to an increased excretion of iodine in the urine.

In Table II is shown the ratio of isotopic to stable iodine in plasma or urine before and at two to four hours after operation together with the urinary iodine (I^{127}) excretion during the 24 hr. before operation and during the first 24 hr. following operation.

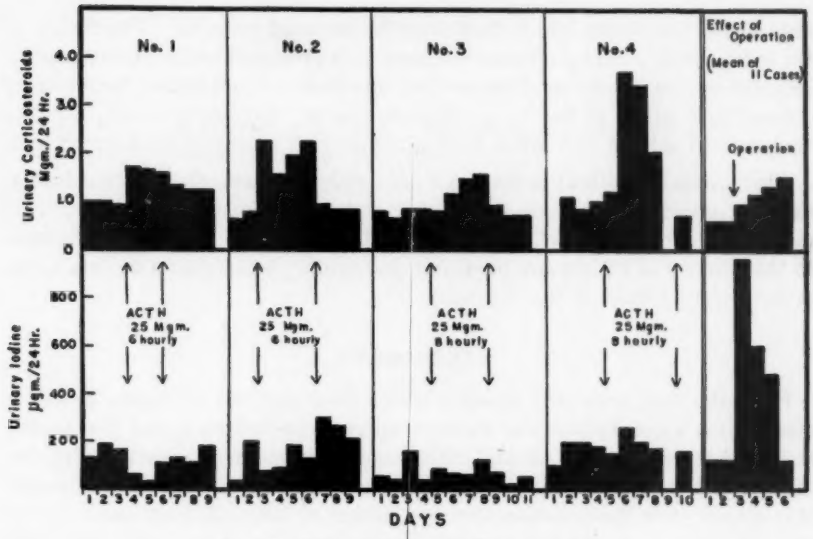

FIG. 1. *Effect of ACTH on iodine excretion.*

TABLE II

INFLUENCE OF OPERATION ON IODINE METABOLISM

Subject	Procedure	Urinary I^{127}, µgm./24 hr.		Ratio I^{131}/I^{127} in plasma or urine		
		Before operation	After operation	Before procedure	2–4 hr. after procedure	% Initial value
5	Operation	45	907	0.38	0.02	4.0
6	Operation	145	105	0.19	0.13	69.0
7	Operation	118	697	0.34	0.01	3.5
8	Operation	156	475	1.10	0.23	20.0
9	Operation	116	2080	0.42	0.02	4.5
10	Operation	206	600	1.50	0.05	3.2
11	I.v. iodide	—	—	3.20	0.01	0.2
12	I.v. iodide	—	—	0.46	0.02	3.3
13	Nil	—	—	0.70	0.30	43.0
14	Nil	—	—	0.38	0.32	85.0

It will be seen that in six operative cases examined five showed a marked diminution of the ratio I^{131}/I^{127} following operation. The one exception was a subject who was also exceptional in not showing a postoperative increase in iodine excretion.

In contrast are two control subjects in whom no procedures were carried out other than determination of the ratio I^{131}/I^{127} at similar time intervals as in the above six subjects. In these two normal subjects the ratio declined

only slightly in comparison with that in the operated subjects. The decline of the ratio which occurs in the normal subject is presumably due to the gradual influx into the iodide compartment of the body of unlabelled iodine from dietary and other sources—e.g. degradation of thyroid hormone. In the traumatized subject this influx from other sources is greatly accelerated.

Also shown in Table II is the result of introducing suddenly into the circulation (by means of intravenous injection) 5 mgm. of unlabelled iodide. This was done in two subjects. The results duplicated the effects of operation in that there was an increase in plasma and urinary iodine but a decline in the ratio I^{131}/I^{127} following the injection.

Discussion

From the data presented above it seems clear that the alteration in iodine metabolism which follows the stress of surgical operations is not due to the increased activity of the adrenal cortex and consequent increased production of cortical hormones which also takes place as a result of trauma. It would also appear that the postoperative production of iodine is from some source other than the inorganic iodide of the body tissues, i.e., from some unlabelled source, otherwise the ratio I^{131}/I^{127} would have exhibited only a slight decline similar to that observed in the normal subjects.

It is conceivable that the post-traumatic increase in iodine excretion is a result of an increased secretion of thyrotrophic hormone mobilizing iodine from the thyroid gland and also possibly a result of an accelerated breakdown of thyroid hormone in the peripheral tissues.

It has been shown by various investigations (2, 4) using histological and radioiodine techniques that the stress of cold brings about an increase in thyroid activity due to an elaboration of thyrotrophin. In addition Williams *et al.* (5) have induced thyroid hyperactivity by means of other stress situations as well as cold and at the same time have found evidence which indicates that stress is followed by an increased degradation of thyroid hormone peripherally. Further support of this latter concept is perhaps found in the elevation in metabolic rate which occurs following operations (1). Whether the post-operative increase in iodine excretion is a consequence of thyrotrophin secretion or a consequence of an accelerated breakdown of thyroid hormone, or both, remains to be investigated.

Acknowledgments

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THE FREE-CELL PHENOMENON IN ISOHAEMAGGLUTINATION[1]

By Kenneth W. McKerns and Orville F. Denstedt

Abstract

An hypothesis is presented to account for the invariable persistence of unagglutinated red blood cells in agglutinated samples even when potent isoagglutinating serum is used. It is postulated that the cells which remain free represent the few which become completely saturated with the agglutinin during the sensitization phase of the reaction. These cells, which amount to only a fraction of a per cent of the total originally present in the sample, are incapable of reacting with one another but can undergo agglutination with fresh cells of the same blood type thus indicating that they are morphologically normal. Evidence is presented in support of the hypothesis. The free-cell count is reproducible provided the agglutinating serum is potent and the conditions of the experiment are standardized. The influence of various factors on the free-cell count, and the usefulness of the count for following changes in the avidity of isoagglutinating serum with time, are discussed.

When erythrocytes are exposed to a specific agglutinating antiserum a few cells invariably escape agglutination even with highly potent sera. These so-called "free" or "unagglutinable" cells amount to a fraction of a per cent of the total number in the sample. The free-cell count may be reproduced with accuracy provided potent antiserum is used and the conditions such as the temperature, size, and shape of the container, rate of agitation, and others are kept constant. The failure of some of the cells to be agglutinated is of no consequence in qualitative tests such as blood-typing but, in certain quantitative procedures, the free-cell count may constitute the basis of the method. It is necessary in such cases to eliminate the possibility of variation due to changes in the conditions of the test. For example, in the Ashby method of differential agglutination for following the rate of elimination of the donor's cells from the recipient's circulation after transfusion, the free-cell count of the recipient's blood must be determined prior to the transfusion, and the conditions of agglutination must be standardized throughout the investigation.

Among the factors which strongly influence the free-cell count is the strength of the agglutinating serum (10). The strength or "potency" usually is expressed in terms of the "titer" and the "avidity". The former represents the maximum dilution of the serum that will produce specific and visible agglutination of a cell suspension or a colloidally dispersed antigen. However, it affords only a relative indication of the actual concentration of antibody since the value obtained will vary depending on the antigen content of the cells used, the duration of the test, the electrolyte concentration, temperature,

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Contribution from the Department of Biochemistry, McGill University, Montreal, Que.

This study represents part of a dissertation presented by K. W. McKerns in September 1949 in partial fulfilment of the requirement for the degree of Doctor of Philosophy at McGill University. A review of the study was presented at the meeting of the Committee on Blood and Blood Derivatives of the National Research Council in Washington, D.C. on July 8, 1949. The principal points of the study were reported also at the annual convention of the Canadian Physiological Society in Montreal on October 15, 1949.

and the rate of collision and intimacy of contact of the cells. It is well known that when the estimation is carried out in the test tube with centrifugation the titer value obtained may be from 6 to 10 times that given by the slide method in which the cells are brought in contact with one another by simple agitation. The relative nature of the titer is brought out in DeGowin's study in which titer determinations were made on a number of given isoagglutinating sera in several laboratories (5). The titer method, while adequate for testing the acceptability of antisera for routine blood-typing, is not entirely satisfactory for the quantitative evaluation of the antibody concentration in isoagglutinating sera.

The term "avidity" also is an indefinite one which implies the rapidity of action of an antiserum. This property apparently has more to do with the sensitization phase than with the aggregation phase of the agglutination reaction. It represents the affinity of antibody for antigen and thus reflects the rate of combination of the two. Normal anti-A and -B sera, and heterologous immune agglutinating sera such as anti-M and -N, when fresh, usually exhibit strong avidity. There appears to be no relation between this property and the titer. Certain antisera, notably anti-M and -N, may show strong avidity at a relatively low titer while, with certain others, the reverse relationship may exist. Pillemer (11) studied the two properties in various antisera and in isoagglutinin globulin fractions, and tested the effect of dilution, extraction of the lipid, and other treatments on the potency. He presents evidence that both a lipoprotein complex and a "heavy protein" are essential factors in the agglutinating complex, and that avidity is related to the lipoprotein. Studies on the assay of the potency of isoagglutinating sera have been reported also by Boyd (1), Oncley *et al.* (9) and others (8, 12). In these studies the estimation of potency is based on the titer, and on the avidity according to the respective times when agglutination becomes perceptible and when it reaches the maximum as judged by the appearance of the cell clumps.

In the study reported herein an effort has been made to characterize avidity on the basis of the free-cell count.

The reason why some of the red cells in any sample of blood fail to be agglutinated has received little systematic study. The only reports we have been able to find on the subject are those of Callender *et al.* (3) and of Young *et al.* (15). The former group found that the free-cell count is not influenced by the proportion of reticulocytes in the blood. The latter group of investigators showed that the reticulocytes are agglutinated in the same manner as mature cells and that the erythrocytes which fail to be agglutinated are not morphologically abnormal.

In the course of our study on the influence of various factors on the agglutination of erythrocytes an effort was made to ascertain the cause of the unagglutinable cell phenomenon. At first the following factors appeared to be among the plausible ones to be considered:

(1) Failure of some of the cells to come in intimate contact with others.

(2) Insufficiency of antibody to produce complete agglutination.

(3) Absence of agglutinogen on the free cells, or perhaps, the presence of weakly reactive agglutinogen.

The first of these explanations is ruled out by the persistence of free cells even after repeated centrifuging of the sample and resuspension of the cell sediment. The second also can be eliminated by two observations, namely, (*a*) that the free-cell count cannot be reduced significantly by the further addition of antiserum, and (*b*), that when potent antiserum is used in the ordinary agglutination test there always is a sufficient excess of antibody to cause the agglutination of several further additions of cells.

The third explanation is more difficult to test, particularly with regard to the reactivity of the agglutinogens. However, it can be shown that the free cells are not devoid of agglutinogen, for, while they are unagglutinable among themselves, they are capable of reacting with fresh cells. The latter discovery shed new light on the problem and suggested a fourth explanation which is supported by a substantial body of evidence as set forth in the remainder of this paper.

The hypothesis to be described is based on the idea that the red cells may undergo various degrees of saturation with antibody. In accordance with present-day concepts the agglutinins are assumed to be at least divalent. In the initial, or sensitization phase of the agglutination reaction, agglutinin combines with a specific agglutinogen on the cells. In the second, or aggregation phase, the cells, on contact, become linked together by agglutinin and precipitate out in clumps. Both stages of the reaction appear to be specific (7). The hypothesis, to be presented, postulates that the great majority of the cells suffer collision and aggregation before sensitization is complete, while a few become completely saturated with antibody before making adequate contact with other cells. The saturated ones constitute the so-called free or unagglutinable cells. Since all their receptor sites are occupied these cells are incapable of undergoing aggregation among themselves. Another small proportion of the cells may approach saturation and subsequently either become saturated or undergo agglutination. Thus the free-cell count tends to fall exponentially to a virtually constant value on prolonged agitation or on repeated centrifugation of the sample.

From the foregoing hypothesis one may anticipate the following results:

(1) that cell saturation should be favored by a high concentration of avid antibody. In other words, the free-cell count should be relatively high with a potent antiserum.

(2) that a "zone" effect may be expected with highly potent antisera; in other words, optimal agglutination should be obtained only after appropriate dilution of the serum.

(3) that the number of free cells should be greater when agglutination is carried out at higher temperatures, at least up to about 40° C.,

(4) that the free cells should be capable of reacting with fresh, or incompletely saturated cells,

(5) that the free cells obtained on agglutination at one temperature should be incapable of reacting with those formed at another temperature, since all should be saturated,

(6) that it should be possible to demonstrate the presence of antibody on the free cells by means such as the Coombs test (4).

Confirmation of these inferences has been obtained.

Experimental

1. *Agglutination Procedure*

A red-cell suspension is prepared by drawing blood up to the 0.5 mark in a white-cell pipette. The latter then is filled to the 11 mark with isotonic saline (0.9% sodium chloride) and the contents, after being mixed by agitation, are transferred to a small serological test tube. Into a dry white-cell pipette the cell suspension is drawn up to the 1.0 mark and the pipette then filled to the 11 mark with the temperature-equilibrated agglutinating serum.* The contents are mixed and transferred to a serological tube which then is placed in a shaker and agitated at a constant rate (230 vibrations per minute**), for 15 min. in a water bath maintained at a constant temperature (20° C.). The tube is centrifuged at 1500 r.p.m. in a table-model machine for two minutes. After the cell sediment is broken up and suspended by flicking the tube, the contents are again centrifuged. The tests are performed in duplicate and the free-cells counted in a hemocytometer. The counting is done with a medium objective and care is taken to avoid counting the white cells which may be present. The number of free cells per cu. mm. is obtained by multiplying the count taken over the entire field by the factor 269.

2. *Reproducibility of the Free-cell Count*

The number of free cells will vary with changes in the conditions of agglutination but, provided the conditions are kept constant, the count can be reproduced with accuracy. Table I illustrates the agreement obtainable under standardized conditions.

3. *Variation in Free-Cell Count Over a Period of Time*

Table II illustrates the variation of the free-cell count in fresh red-cell specimens removed periodically from three donors by finger puncture. The data on Specimen 1 were obtained in a previous study (10).

In Table III are given the free-cell counts on two lots of preserved red cells (whole blood). The specimens were preserved in citrate–glucose and kept at 5° C. Periodically samples were removed aseptically for the tests.

* *Prior to being used the serum is agitated for 30 min. in a water bath at the desired temperature.*

** *A specially designed shaker is used, equipped with a Graham variable speed motor and a bath which may be set at various temperatures. The length of stroke of the shaker also may be adjusted.*

TABLE I

REPRODUCIBILITY OF FREE-CELL COUNT

Test No.	Specimen 1, in duplicate Cells: Group B Serum: anti-B Titer: 256 Dilution 1:1		Specimen 2, in duplicate Cells: Group B Serum: anti-B Titer: 256 Dilution 1:1	
1	7250	6400	16,150	17,200
2	7000	6450	15,350	15,900
3	6200	8050		
4	7250	7550		
5	6700	7250		
6	6400	7800		
	Mean 7025 ± 97 (S.E.)		Mean 16150 ± 525	

Tests on Specimen 1 carried out at 20° C.—agitated for 30 min. at 230 vibrations per min.
Tests on Specimen 2 carried out at 20° C.—agitated for 60 min. at 230 vibrations per min. with two centrifugations.

TABLE II

FREE-CELL COUNT ON FRESH RED CELLS

Day of test	Specimen 1 Cells: Group A Serum: anti-A Titer: 5000 Diluted: 1:5	Specimen 2 Cells: Group B Serum: anti-B Titer: 256 Diluted: 1:1	Specimen 3 Cells: Group B Serum: anti-B Titer: 256 Diluted: 1:1
0	14,500		
1		22,600, 22,150 — 22,375	16,600, 15,900 — 16,250
3		10 a.m.: 10,200, 10,500 — 10,350	6190, 6450 — 6350
		3 p.m.: 9700, 9950 — 9825	6750, 7200 — 6975
7	11,000	15,600, 14,550 — 15,075	12,100, 13,400 — 12,750
10		14,250, 15,300 — 14,775	10,700, 9700 — 10,200
14	22,000		
18		6700, 6450 — 6575	4300, 4800 — 4550
21	23,500		
28		9400, 9950 — 9675	8600, 8350 — 8475
35	12,500		
43	16,000		
49	8000		

Anti-B serum, titer 256, diluted 1:1 with isotonic saline agitated 30 min. at 230 vibrations per min. and twice centrifuged and resuspended.

It will be observed that there was no difference in the magnitude of the variation in the free-cell count in the fresh and the preserved samples.

TABLE III

FREE-CELL COUNT ON PRESERVED RED CELLS (10)

Day of test	Specimen 1 Cells: Group A Serum: anti-A Titer: 5000 Diluted 1:5	Specimen 2 Cells: Group B Serum: anti-B Titer: 5000 Diluted 1:5
0	12,500	9000
2		12,500
5		11,500
7	19,000	11,000
9		6500
12		5500
14	11,000	
17		7500
19		9500
21	15,500	8000
23		10,500
26		9000
29		11,000
33		7000
35	15,000	
40		9500
43	11,000	
44		5000
47		10,500
49	31,000	
53		12,000
61		4500

It is evident that the free-cell count with given antisera and red cells may fluctuate to a considerable degree over a period of time. In a previous study (10) evidence was obtained that the fluctuations were attributable to a reversible type of change in the antisera rather than to an alteration in the cell agglutinogens. This is further confirmed by the results in the two experiments in Table II, in which the same antiserum was used concomitantly with the cells of two group-B individuals. The fact that the fluctuation in the free-cell counts in the two cases occurred at the same time and in the same sense again suggests that the antiserum, and not the cells, was the responsible factor.

4. Influence of Dilutions of the Serum on the Free-Cell Count

With potent antisera the rate of aggregation usually is more rapid, and the size of the clumps tends to be larger in some of the diluted samples than with the undiluted serum. This frequently is seen in titer tests. To obtain the minimum free-cell count, it is necessary to dilute the serum. Obviously the minimum free-cell count affords a more reliable criterion of the completeness

of agglutination than does a judgment of the size of the clumps. Table IV illustrates the fall in the free-cell counts on dilution of various high-titer anti-A sera with isotonic saline.

TABLE IV

INFLUENCE OF DILUTION OF THE SERUM ON THE FREE-CELL COUNT

(Free-cell counts per cu. mm. of various dilutions of the sera)

Serum sample	Titer	Series of dilutions						
		Undiluted	1:1	1:2	1:5	1:10	1:20	1:40
1	2048	98,400				80,500	16,100	
2	2048				62,100	59,600	50,500	
3	512			22,000			7800	
4	2048			70,200			22,400	10,750
5	1024		23,400				13,700	

The same Group A cells were used in all the tests.

5. Free-cell Count after Repeated Addition of Fresh Cells

The following experiment illustrates the tendency of the free-cell count to remain practically unchanged when repeated agglutinations are carried out with a given amount of a potent antiserum, i.e., with a large excess of antibody initially present, and on repeated addition of red cells.

Procedure

A suspension of red cells (ANRh) was prepared by drawing whole blood up to the 0.5 mark in a white-cell pipette and then filling the latter to the 11 mark with isotonic saline. After shaking the pipette the contents were transferred to a small serological tube. With the same pipette (washed well and dried before each use) the cell suspension was drawn up to the 1.0 mark, and transferred to each of three serological tubes, A, B, and C. A fourth aliquot of suspension was pipetted to the 1.0 mark and the pipette then filled to the 11 mark with the agglutinating serum. The thoroughly mixed contents were transferred to a serological tube, D (kept at 25° C.) and then placed in the shaker for agglutination in the manner previously described. In the meantime the cells in Tubes A, B, and C were centrifuged for two minutes at about 1500 r.p.m. and the supernatant fluid was then removed carefully with a capillary tube. The three tubes, containing the sedimented cells, were placed in a water bath at 25° C. for temperature equilibration. After the prescribed agglutination period the agglutinated cells in Tube D were permitted to settle for two or three seconds and the supernatant fluid containing the free cells was transferred to Tube A*. The cells were suspended by flicking the tube and then placed on the shaker for agglutination. After the usual period,

* *In some of the experiments the pipette was filled from the supernatant portions of duplicate agglutinated samples in order to increase the accuracy in making the transfer of the free cells.*

the free cells from A were transferred to Tube B and the agglutination repeated. In a similar manner the free cells from B were added to C. By this procedure the volume of the samples and the conditions of treatment were kept nearly constant during the four runs. The experiment was repeated several times using different antisera.

TABLE V

CONSTANCY OF THE FREE-CELL COUNT ON REPEATED AGGLUTINATION OF CELLS WITH A GIVEN VOLUME OF POTENT ANTISERUM

Experiment	Antiserum			Free-cell counts per cu. mm. in the series				
	Type	Titer	Dil'n	I (1 vol. cells)	II (2 vol.)	III (3 vol.)	IV (4 vol.)	Mean
1	Anti-N	12	Undil'd	12,900	12,700	14,000	13,300	13,325 ± 298
2	Anti-A	512	"	6200		6650		6425 ± 225
3	Anti-B	128	"	10,200		10,100		10,150 ± 50
4	Anti-M	64	"	17,200		18,300		17,750 ± 550
5	Anti-N	64	"	12,100		13,400	14,800	13,433 ± 729
6	Anti-A (α)	256	1 : 5	33,600		40,000		36,800 ±3200
7	Anti-A(α_1)	128	1 : 1	11,050		12,100		11,575 ± 525

The results of typical experiments are given in Table V. In Experiments 2 to 7 the free-cell count in the initial run was determined in the usual manner, i.e. the supernatant fluid containing the free cells was added to two volumes of sedimented normal cells. The mixture was agglutinated in the usual way.

Thus, when a large excess of antibody is present, the free-cell count remains remarkably constant on successive additions of fresh cells. It is evident that most of the free cells from each test must have undergone agglutination with fresh cells in the subsequent one. This was demonstrated further by labelling free cells with sulphaemoglobin (7) and then adding them to a suspension of fresh cells. On examining the agglutinated cell sediment under the microscope the clumps were observed to consist of a mixture of labelled and unlabelled cells. A control test, using the sulphurated free cells alone with the supernatant fluid from the previous agglutination, produced no agglutination.

6. Influence of Temperature on the Free-cell Count

Any factor which influences the rate of the reaction between antigen and antibody should be expected to influence the incidence of cell saturation. It may be anticipated, therefore, that the free-cell count will tend to be greater at higher temperatures, at least up to about 40° C. Above the latter temperature the results may be expected to become erratic because of denaturation of the proteins in the cells and the serum.

Experiments were performed with various anti-A, -B, -M, and -N sera at temperatures ranging from 5° to 40° C. The samples were shaken for 20 min. at 230 vibrations per minute, twice centrifuged, and the cell sediments

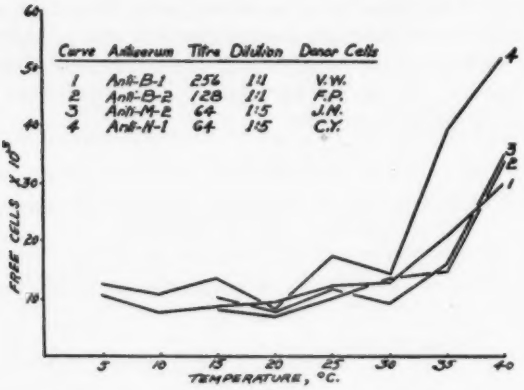
Curve Antiserum Titre Dilution Donor Cells
1 Anti-B-1 256 1:1 V.W.
2 Anti-B-2 128 1:1 F.P.
3 Anti-M-2 64 1:5 J.N.
4 Anti-N-1 64 1:5 C.Y.
FREE CELLS X 10⁵
TEMPERATURE, °C.

FIG. 1. *Influence of temperature on the free-cell count obtained with avid isoagglutinating sera.*

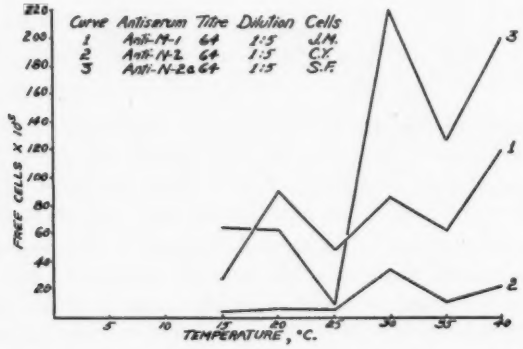

FIG. 2. *Influence of relatively weak avidity on the free-cell count obtained at various temperatures.*

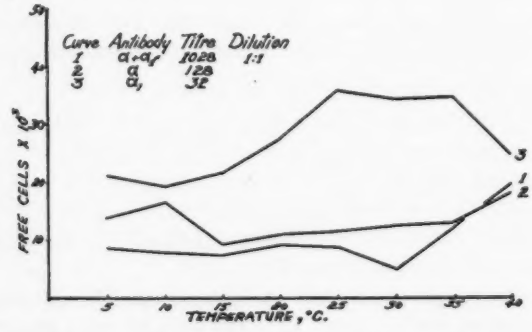

FIG. 3. *Influence of temperature on the free-cell count obtained with the α and α_1 isoagglutinins before and after separation.*

resuspended after each centrifugation. The free-cell counts obtained are indicated by the curves in Figs. 1, 2, and 3.

The comparatively regular curves in Fig. 1 represent the free-cell counts obtained at various temperatures with sera of relatively high avidity. The less regular curves in Fig. 2 are typical of those with less avid sera (10, 11). The particular anti-M and anti-N sera used in the latter tests had been in storage for several months. Furthermore, it was necessary to dilute them to ensure a sufficient supply for the experiment.

Changes in temperature between 5° and 30° C. appear to have a relatively slight effect on the free-cell count with avid anti-A, -B, -M, and -N sera. Between 30° C. and 40° C., however, the count tends to be considerably increased and the size of the clumps smaller with increase in the temperature. These observations suggest that the cohesion of the aggregated cells may be weaker at the higher temperatures and that some of the cells may be shaken free from the clumps and perhaps may become saturated.

In view of the composite nature of anti-A sera, one might anticipate a peculiar type of behavior of the free-cell count with these sera depending on the concentration and avidity of the α and α_1 or α_2 antibody constituents. Fig. 3 illustrates the free-cell counts obtained at various temperatures with a potent anti-A serum and with the α and α_1 components after separation (see footnote p. 162). The curve with the α_1 agglutinin suggests that this antibody is relatively sensitive to changes in temperature. The poor recovery of the antibody in the process of isolation accounts for the low titer.

7. Reactivity of Free Cells Formed at Different Temperatures

It is to be anticipated that the free cells formed at different temperatures should not undergo agglutination when mixed since presumably all are saturated. This inference was confirmed in several experiments of which the data given in Table VI are representative.

TABLE VI

MIXTURE OF FREE CELLS FORMED AT DIFFERENT TEMPERATURES

Sample	Temperature used in agglutination test	Free cells per cu. mm.		
		Experiment 1	Experiment 2	Experiment 3
(*a*)	20° C.	6450	5370	6450
(*b*)	30° C.	12,900	25,200	29,000
(*c*) Found	20° C.	8870	15,100	16,700
Expected		9675	15,285	17,850

The sample (*c*), consisting of equal volumes (the usual quantities) of the supernatants (containing the free cells) from (*a*) and (*b*), in each experiment was shaken at 20° C. Thus the observed free-cell count was close to the expected count, namely half the sum of the counts in (*a*) and (*b*).

In Experiment 1 cells of Group ANRh were used; in Experiment 2, Group ONRh, and in 3, ANRh. The same anti-N serum (titer 64, diluted 1 : 5) was used in the three experiments.

That the free cells are not devoid of reactivity was demonstrated in another experiment in which the supernatant serum, containing the free cells from one agglutination test at 30° C., was mixed with the prescribed quantity of fresh cells, and the mixture agglutinated at 20° C. The new free-cell count was compared with that in a sample which had been agglutinated at 20° C.

Procedure

Two lots of cells (ANRh) were prepared for agglutination in the usual manner and were agitated at 230 vibrations per minute for 50 min., one of the samples (*a*), at 30° C. and the other (*b*), at 20° C. In either case the specimen, after the period of agitation, was centrifuged for two minutes at 1500 r.p.m. The cells were resuspended, the tube again centrifuged, and the cells resuspended. After determining the free-cell count the supernatant (containing the free cells) from Specimen *a*, was added to the sedimented fresh cells (saline having been removed) in a sample (*c*) and the suspension agglutinated at 20° C.

The free-cell counts in the three samples are given in Table VII.

TABLE VII

FREE CELLS OBTAINED AT 30° C. AGGLUTINATED WITH FRESH CELLS AT 20° C.*

Sample	Temperature of test	Free cells per cu. mm.		
		Series (1)	Series (2)	Series (3)
(*a*)	30° C.	23,600	19,400	29,000
(*b*)	20° C.	10,500	8600	6450
(*c*)	20° C.	10,500	9400	6700

* *Cells ANRh; serum anti-N, titer 64, diluted 1 : 5.*

In Series 1, red cells of Group ANRh were used with anti-N serum (titer 64, diluted 1 : 5), and in series 2, the same donor's cells with anti-A (α) serum* (titer 64, diluted two times). The agreement between the counts in (*b*) and (*c*) is close and indicates clearly that the free cells from the agglutination at 30° C. must have reacted with the fresh cells in the subsequent test.

To test whether any alteration is produced in the reactivity of the cells or the serum at 40° C. the red cells and the serum separately were heated at 40° C. for 30 min. On repeating the agglutination test with these materials

* *The serum was prepared according to the procedure of Landsteiner and Miller (13). Anti-A (α and α_1) serum was absorbed repeatedly with Group A_2 cells until no further reaction was obtained with the cells. The agglutinated cells from the successive trials were suspended in a small volume of isotonic saline and warmed at 56° C. for two minutes. The sample then was centrifuged for two minutes at the latter temperature and the supernatant fluid removed. The titer of the released α agglutinin was found to be 64.*

at 20° C. the free-cell count was found to agree with that obtained with unheated cells and serum. At higher temperatures, however, the results tend to become erratic.

8. Reaction of Saturated Cells with a Different Antibody

The question arose whether cells, after saturation with one antibody, are capable of reacting with a different antibody. The following experiments indicate that a second and perhaps a third reaction can occur.

Red cells of Group AMNRh were agglutinated at 20° C. with anti-M* in one tube, and with anti-N* in another. The free-cell counts in the two samples were respectively 32,250 and 15,100 per cu. mm. The contents of the two tubes then were mixed, twice centrifuged, resuspended, and examined. The free-cell count was found to be reduced to 3225 per cu. mm.

In another experiment cells of Group ANRh, after agglutination separately with anti-A serum, and anti-N respectively, gave a free cell count of 13,450 and 24,310 respectively per cu. mm. On agglutination with mixed anti-A and anti-N sera the free-cell count was found to be 7260 per cu. mm.

These observations confirm the evidence obtained in another phase of our study, namely, that the different agglutinogens appear to be distributed in patches on the red cell surface and that the saturation of one antigen with the corresponding antibody appears not to interfere greatly with the reactivity of the other antigens.

9. Estimation of Avidity of Antisera

a. Characterization of Antisera

The following three criteria have been used in our study for evaluating the potency of antisera:

(1) Titer, with, and without, centrifugation.

(2) Free-cell count under constant conditions of agglutination.

(3) The free-cell count at specified times during the period of the test.

The first of these affords a rough index of the antibody concentration; the second indicates the number of saturated cells which, in turn, reflects the avidity of the serum, i.e. the rate of combination of antigen with antibody; the third also reflects the rate of sensitization and thus the avidity. With avid sera, saturation of the cells occurs rapidly, and the free-cell count tends to reach a constant value within a relatively short time. Thus the rapidity with which the count reaches a constant level, rather than the magnitude of the final count, is the important criterion in the test.

To obtain a comparable base of reference for each serum the dilution was chosen which gave the lowest free-cell count. This was determined by the following procedure using a 15 min. period of shaking since, with our apparatus, the reaction is virtually complete within 15 min.

* *The anti-M serum previously had been absorbed with Group-N cells and the anti-N with -M cells.*

Procedure

Red cells, containing the appropriate agglutinogen, were agglutinated with the antiserum by shaking the sample for 15 min. at 20° C. at 230 vibrations per minute. The tubes then were centrifuged twice at 1500 r.p.m., each time for two minutes. The clumps were broken up after each centrifugation by sharply flicking the tube. This procedure was repeated for the various dilutions in the titer series and the dilution which gave the lowest free-cell count was noted. A quantity of the original serum, sufficient for the final test, was prepared in this dilution.

In the avidity test three samples of cells and diluted antiserum, which may be designated (*a*), (*b*), and (*c*) respectively, were prepared in the usual way and were treated in the following manner:

(*a*) Sample shaken for one minute at 20° C. and then centrifuged for one minute.

(*b*) Sample shaken as in (*a*) but with two centrifugings of two minutes duration, the cell sediment being resuspended after each centrifugation.

(*c*) Sample shaken for 15 min. at 20° C., followed by centrifuging twice for two minutes and resuspension of the cells after each centrifugation.

The free-cell counts obtained in several experiments of this kind are given in Table VIII.

TABLE VIII

FREE-CELL COUNTS AFTER THREE TREATMENTS AT 20° C.

Antiserum	Titer	Serum dilution used	Free-cell counts per cu. mm.		
			Sample (*a*)	Sample (*b*)	Sample (*c*)
Anti-A (α and α_1)	2048	1 : 1	26,600	12,600	6100
Anti-B	256	1 : 1	14,250	10,500	10,200
Anti-M	64	1 : 5	54,000	11,200	6700
Anti-N	64	1 : 5	15,900	6300	6200
Anti-A (α)	128	1 : 5	22,600	14,300	10,700
Anti-A (α_1)	64	1 : 4	22,600	38,000	29,600

It will be observed that the free-cell count reached a constant value most rapidly with the anti-B and the anti-N sera thus indicating that these were the most avid of the series. The anti-M serum was a sample which had been stored in the cold for several months. With the α_1 serum the free-cell count was higher in (*b*) than in (*a*) owing to disintegration of the clumps on continued agitation. Thus an increase in the count may occur if the cells in the aggregates are very weakly bound together; a simple flicking of the tube in some instances, e.g. in Rh agglutination, may release individual cells from the aggregates. While this serum showed a low avidity, the behavior is not a characteristic of anti-A sera as a rule.

b. Decrease in Avidity of Antiserum with Time

The avidity of serum may undergo a gradual decrease during storage particularly if the material has been repeatedly frozen and thawed. The deterioration may be accelerated by unfavorable conditions of storage. Table IX illustrates the fall in avidity of one anti-A serum during storage at 5° C. The original titer of the serum was 2048 (at 20° C. tested with red cells of type ANRh), and appeared to be unchanged at the 25th day of storage when tested with the same cells. However, a slight decrease was detected in the titer of the α_1 agglutinin in special tests.

TABLE IX

DECREASE IN AVIDITY OF AN ANTI-A SERUM WITH STORAGE

Dilution of serum	Free-cell count per cu. mm.	
	With serum when fresh	Serum after storage 25 days
1 : 2	72,300	8600
1 : 20	22,400	10,200
1 : 40	10,750	12,600

Shaking period 15 min., followed by two centrifugations

It will be observed that the free-cell count with the fresh serum was high in the 1 : 2 dilution and fell to 10,750 in the 1 : 40 dilution. After storage for 25 days the number of saturated cells in the 1 : 2 dilution was comparable with that of the 1 : 40 dilution of the fresh material. The counts in the 1 : 20 and 1 : 40 dilutions in the 25-day specimen represent an incomplete reaction.

Discussion

In our study, instead of evaluating the avidity of isoagglutinating sera on the basis of the respective times when agglutination becomes perceptible and when it reaches the maximum as judged by the appearance of the clumps, we have based our estimate on the free-cell count. The criteria used were: (*a*) the degree of dilution of the serum necessary to give the lowest free-cell count, and (*b*) the free-cell count at the end of 15 min., by which time the count reaches a practically constant value under the condition of the test. By both the older procedure and ours, in order to obtain reproducible results it is necessary to standardize the various conditions of the tests such as the volume of the sample, the proportion of cells and antiserum, temperature, and the rate and mode of agitation. Because of the variability of the results with the conditions, the values obtained have a relative rather than an absolute significance, and thus, the methods are of limited value for purposes of standardization. The free-cell method offers the advantage that the values are based on an accurate count rather than on the judgment of the worker. Furthermore, the ability to define the conditions of the procedure

makes possible the duplication of the results in any laboratory. The chief value of the method, however, lies in its usefulness for following changes in the avidity of antisera during storage and other treatments, and in studying the antigen content of the red cell. Its usefulness will be extended when more is known about the nature and absolute concentration of the isoagglutinins in the serum and the agglutinogens in the cells.

The saturation concept appears to account for the free-cell phenomenon and certain others which hitherto have not been satisfactorily explained. Also, it fits in with current ideas on the mechanism of agglutination whether the process be viewed from the standpoint of the law of mass action, or as an adsorption phenomenon (14, 2). Further studies on the kinetics of the process would be facilitated by a clearer understanding of the chemical basis of avidity and the mode of distribution of the various antigens on the cell surface. There is little evidence that the reaction between agglutinogen and agglutinin is reversible to any considerable extent at temperatures below 40° C. At considerably higher temperatures in the region where denaturation of the cell and plasma proteins occurs, a partial release of antibody can be effected.

The observed day-to-day fluctuation in the free-cell count with given red cells and potent agglutinating sera has not yet been satisfactorily explained. It has been shown that the free-cell count, when carried out under controlled conditions, is reproducible within about 5–8%, depending on the cells and the antiserum, provided the tests are performed about the same time, i.e. on the same day. When repeated at intervals over a period of several days or weeks, on the other hand, the count may undergo a fairly large day-to-day variation which apparently is not due to loss of potency. The observation that the degree of variation was found to be the same for fresh and for efficiently preserved red cells in concurrent tests on a given donor's blood, suggests that the cause lies in the antiserum and not in the cells. In a previous study (10) as well as in that reported here, we obtained evidence that the isoagglutinin, or some other influential component of the antiserum, is capable of undergoing a reversible type of change which appears to influence the avidity of the antibody. This behavior, furthermore, is accentuated when the antiserum is diluted, even though the material may still be of high titer and potency. An effort was made in our study to eliminate these changes by storing the sterile antiserum in small sealed tubes at 5° C., and equilibrating it at the time of use by agitating it in a water bath for 30 min. at the desired temperature. Despite these and other precautions the periodic variation in the free-cell count was found to persist.

The periodic fluctuation of the free-cell count of the recipient's blood does not seriously affect the usefulness of the Ashby method for following the elimination of the donor's cells after transfusion. During the early period of the experiment, when the total number of free cells may be from 200,000 to 500,000 per cu. mm. depending on the quantity of blood transfused (200 to 500 ml.), the variation in the recipient's free-cell count is of little significance.

Thereafter, and until the experiment is completed, the only effect of the variation is to produce a slightly irregular, rather than a smooth cell-elimination curve. Finally, when all the donor's cells have been eliminated, the curve levels off at the recipient's free-cell count. If the agglutinating serum is fairly fresh at the commencement of the tests, and is stored under sterile conditions at 5° C., it is unlikely that serious deterioration of the potency will occur (at least with anti-A or anti-B) during the four-month period of the experiment.

With the occasional highly potent anti-A agglutinating serum of the hyperimmune type it is almost impossible to obtain closely reproducible free-cell counts in successive agglutinations (as described on p. 159, Table V) even when the serum is suitably diluted. This observation leads one to suspect that unusual factors sometimes may be present, such as A-blocking antibodies the occurrence of which has been reported by Hubinont (6).

That the titer values obtained with a given isoagglutinating serum and the cells of various individuals of the same blood group or type may differ quite widely has long been known. This observation suggests that the concentration and distribution of the agglutinogen in question may be quite different from one blood to another. It is generally accepted, for example, that homozygous cells, e.g. AA, contain more of the antigen A and thus exhibit a stronger reaction towards anti-A than do the heterozygous AO cells. There are few individuals, if any, with the exception of identical twins, who are identical with respect to kind, content, and distribution of the red-cell agglutinogens, and thus it is understandable why the free-cell count with a given antiserum should differ with individual bloods. Since the free-cell count is partly a function of the agglutinogen content of the cells it is clear also why the count and the titer have little absolute significance for evaluating the antibody content of the serum.

An effort has been made to obtain information regarding the mode of distribution of the agglutinogens on the surface of the erythrocyte. However the different agglutinogens may be distributed, whether in a uniform pattern or mosaic, or in patches, it is significant that while the free-cells do not react to any considerable extent among themselves, the count will drop abruptly if a different antiserum be added, e.g. if after having determined the free-cell count with anti-A serum, anti-M be added. The apparent facility with which the second antibody reacts suggests that previous occupation of the A-sites with anti-A does not make the M-sites inaccessible to anti-M. A further report will be published on this phase of our study.

The number of free cells is relatively high with ordinary anti-Rh agglutinating sera which usually are not of high potency. Because of the weak cohesion between the cells in the aggregates many of the cells may become dispersed again on agitation of the sample. Owing to the unavailability of potent sera of this class it has not been possible for us to carry out a systematic study on the Rh–anti-Rh system.

Acknowledgments

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A STUDY OF HUMAN HYPERSENSITIVITY TO COMPOUNDS OF THE MUSTARD GAS TYPE[1]

By A. M. Moore and J. B. Rockman

Abstract

A method has been developed for expressing quantitatively the results of measurements of human skin sensitivity to compounds of the mustard gas type. Using this method a study has been made of induced hypersensitivity to *bis*(β-chloroethyl)sulphide, and to ethyl *bis*(β-chloroethyl)amine, and of cross reactions involving these two compounds. The relationship between hypersensitivity and the "flare reaction" has been investigated, and a simple method of inducing the latter is reported. A possible significance of these findings in connection with the healing of mustard gas burns is suggested.

Many simple chemical compounds are known to be capable of inducing hypersensitivity in man and in laboratory animals. The extensive work of Landsteiner and his associates (10–14) using citraconic anhydride and picryl chloride, and the work of other investigators (7, 8, 17) with various aromatic nitro compounds has clearly indicated that a property common to such chemical allergens is their ability to react with tissue proteins with the formation of conjugates which behave somewhat like synthetic antigens in that they exhibit a specificity determined by the prosthetic group.

The present paper contains the results of experiments, using volunteer human subjects, in which the sensitizing agents employed have been two compounds of the mustard gas type, viz., *bis*(β-chloroethyl)sulphide, and ethyl *bis*(β-chloroethyl)amine. That the former of these can react with certain proteins was clearly indicated in the work of Berenblum and Wormall in 1939 (2), and has been amply verified in more recent studies (1, 5, 6, 9). It is perhaps not surprising, therefore, that it should have a sensitizing action on human skin when suitably applied. There are several reports on this subject in the literature of the past few years (2, 15). The extreme potency of this type of compound as an allergen is, however, somewhat surprising, and indeed, it must be regarded as an appropriate tool for studying the phenomena connected with hypersensitivity.

The experiments described in the ensuing pages of this report are arranged in four sections. The first deals with the normal range of sensitivity to mustard gas and the effect upon it of various types of exposure to this compound. Part 2 contains the results of similar experiments with one of the "nitrogen mustards". In the third part the question of cross sensitivity toward the two agents is considered. The final section deals with the phenomenon of reactivation, here termed the "flare reaction".

In the following, the abbreviation MG (mustard gas) is used for *bis*(β-chloroethyl)sulphide, and NM (nitrogen mustard) for ethyl *bis*(β-chloroethyl)amine.

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Contribution from Chemical Warfare Laboratories, Ottawa. (Now, Defence Research Chemical Laboratories.)

The word "sensitivity" is used in the special sense of skin sensitivity as measured by the technique described herein, and the term "normal" is used to denote men who had not previously been exposed to either MG or NM.

Methods

The method used in all the sensitivity tests was as follows. Serial dilutions of the compound being tested, in low-boiling petroleum ether, were prepared covering the desired range of concentrations. A single drop of each dilution was allowed to fall freely on to the volar surface of the forearm of the subject from a 27 gauge hypodermic needle, bevel up, on a syringe held at an angle of 45 degrees to the horizontal. This method gave a fairly uniform drop size which was estimated to be 4.50 cu. mm. with a standard deviation of 0.22 cu. mm. The arm was then observed after 24, 48, and 72 hr., and the number of visible reactions recorded. The highest dilution giving a visible reaction during the 72 hr. period was taken as the measure of sensitivity.

If the percentage of men reacting to each dilution is plotted against the logarithm of the concentration, a sigmoid curve is obtained similar to the action curves of many pharmacological agents. Bliss (3, 4) has shown how to transform such data into a linear relationship for convenience in analysis. The procedure he describes has been applied to the data to calculate the regression constants and also the concentration to which 50% of the population react, a quantity which might be termed the "median effective concentration" and which, in the following, is represented by the symbol EC 50.

No attempt was made at the time of application to prevent evaporation of the solute, but it may be assumed that the amount evaporating was a nearly constant fraction of the amount applied, particularly since the area over which the drop spread was approximately the same in all cases. The sensitivity end points are therefore expressed in arbitrary units which, in this case, correspond to the amount of vesicant applied. No estimate has been attempted of the actual amount of penetrated MG or NM required to produce a reaction in the skin.

The MG and NM used in these experiments were carefully purified preparations supplied by the organic chemistry section of Chemical Warfare Laboratories, Ottawa.

Experimental

Part 1—Sensitivity to MG

Group 1—Normal Sensitivity to MG

A group of 60 normal men was tested, as described above, with dilutions of MG in petroleum ether ranging from 1 : 1000 to 1 : 8000 by volume. Forty-five of these were also tested with a dilution of 1 : 500. In this test, as in all the others, the relative positions of the various dilutions being used were randomized with the aid of a table of random numbers. This was done in an attempt to eliminate possible errors due to systematic differences in sensitivity of the skin in different regions of the forearm. The results are given in Table I.

TABLE I

SENSITIVITY TO MG

Log of MG concn.*	Group 1		Group 2		Group 3	
	No. of men	% reacting	No. of men	% reacting	No. of men	% reacting
3.301	45	100	17	88	—	—
3.000	60	90	17	82	14	100
2.699	60	57	17	53	24	96
2.398	60	15	17	47	24	92
2.097	60	0	17	12	24	75
1.796	—	—	17	6	33	54
1.495	—	—	—	—	33	27
1.194	—	—	—	—	33	9
0.893	—	—	—	—	33	3

* *Concentration expressed in cu. mm. MG per liter of solution.*

Group 2—Sensitivity to MG after a Single Burn

Seventeen men were given minor burns by an application of 0.0020 ml. of a 10% (by volume) solution of MG in benzene, delivered by means of a micrometer syringe to each of three spots on the same forearm. (The three burns may be regarded as equivalent to a single larger burn.) Two weeks later test dilutions of MG in petroleum ether were applied to the opposite forearm. The results are summarized in Table I.

Group 3—Sensitivity to MG after Two Successive MG Burns (One Week Interval)

A group of normal men was given three simultaneous burns with 0.0020 ml. of a 10% (by volume) solution of MG in benzene applied to one forearm. Seven days later a second set of three burns was produced by the same means on the same forearm, but at different sites. Seven days after the second set of burns the sensitivity of the men to MG was tested; the results are given in Table I.

In Table III are summarized the results of the probit analysis. The significance of the differences in EC 50 between the foregoing groups has been examined by means of the usual "*t*" test, using as the criterion of significance $P = 0.05$. On this basis the difference in EC 50 between Groups 1 and 2 is not significant, whereas the corresponding difference between Groups 1 and 3 is. A similar test has been applied to the regression coefficient "*b*". Both the differences 1–2 and 1–3 are significant.

Thus it appears that, under the conditions of this experiment, a single MG burn followed by a two week rest period did not result in any appreciable general sensitization of the skin, whereas two similar MG burns seven days apart followed by a seven day rest period produced a definite degree of hypersensitivity.

Part 2—Sensitivity to NM

Experiments similar to those described in Part 1 have been carried out with NM. In all cases the solutions of the free base were freshly prepared just before the tests were made.

Group 4—Normal Sensitivity to NM

A group of normal men was selected for the test. Drops of serial dilutions of NM in petroleum ether were applied, by the technique described under "Methods", to the volar surface of the forearm of each man. The percentage of men reacting to each dilution within the 72 hr. observation period was recorded. The results are given in Table II.

TABLE II

SENSITIVITY TO NM

Log of NM concn.*	Group 4		Group 5	
	No. of men	% reacting	No. of men	% reacting
3.000	21	100	—	—
2.602	38	87	9	100
2.301	38	66	9	89
2.000	48	15	9	67
1.699	48	2	9	44
1.398	48	0	9	44
1.097	—	—	9	22

* *Concentration expressed in cu. mm. per 100 ml. of solution.*

Group 5—Sensitivity to NM after two NM Burns (One Week Interval)

The procedure in this case was similar to that for Group 3 but using NM instead of MG. The burns were produced by the application of 2.0 mgm. pure NM to each of three spots on the forearm. The interval between the first and second sets of burns was seven days. Seven days after the second set of burns was induced the sensitivity was tested on the opposite forearm in the usual way.

Examination of the results of the probit analysis, as summarized in Table III, indicates that the differences in EC 50 and in slope between Groups 4 and 5 are both significant ($P = 0.05$).

Part 3—Cross Sensitivity

Attempts were made to determine whether any cross sensitivity could be demonstrated in the case of men sensitized either with MG or NM. The general procedure was to give a group of men two sets of burns, at an interval of seven days, with one compound, and then, seven days after the second burns, to test their sensitivity with the other compound. In certain experiments sensitivity tests with one or both compounds were made before the

first burns were applied. The results of all these tests are summarized in Table III together with those of the foregoing groups for convenience in comparison.

TABLE III

SUMMARY OF RESULTS OF TESTS WITH MG AND NM

Group No.	No. of men tested	Description of group	EC 50	S.E. of EC 50	Slope of regression line (b)	S.E. of (b)	D.F.
			Log units				
1	45–60	Normal sens. to MG	2.665	0.049	4.014	0.444	2
2	17	Sens. to MG after one MG burn	2.531	0.079	1.878	0.326	2
3	14–33	Sens. to MG after two MG burns	1.777	0.052	2.150	0.241	3
4	21–48	Normal sens. to NM	2.251	0.040	3.692	0.450	1
5	9	Sens. to NM after two NM burns	1.632	0.139	1.455	0.512	2
6	22	Sens. to MG after two NM burns (not previously tested)	2.602	0.081	1.776	0.283	3
7	15	Sens. to MG after two NM burns (previously tested with MG)	2.301	0.093	1.599	0.327	3
8	18	Sens. to MG after two MG burns (previously tested with NM)	1.502	0.117	1.031	0.187	5
9	26	Sens. to NM after two MG burns (not previously tested)	2.225	0.163	2.830	0.488	1
10	18	Sens. to NM after two MG burns (previously tested with NM)	1.824	0.078	1.781	0.312	3

S.E. = *Standard error.*
D.F. = *Degrees of freedom.*
EC 50 = *Concentration to which 50% of group react (calculated) (arbitrary concentration units).*

The ordinate and abscissa for the regression line are "probits" and "log of concentration" respectively.

From the standpoint of cross sensitivity certain of the differences between the tabulated values of EC 50 are of interest. Tests of significance have been applied to these and to the slopes of the calculated regression lines.

The differences in EC 50 between Groups 1 and 6, and between Groups 4 and 9 are not significant. That is to say, two burns with MG, one week apart, did not sensitize these men to NM, nor did two NM burns, one week apart, sensitize them to MG.

The difference between the EC 50's of Groups 6 and 7 is a borderline case but may be regarded as probably significant. This would imply that a simple sensitivity test to MG, consisting of an application of four drops of dilute solutions of MG in petroleum ether, can, at least when coupled with two NM

burns, produce a moderate degree of sensitization to MG. Whether the NM burns had any effect here can not be decided.

The difference in slope between the regression lines for Groups 1 and 6 is significant according to the usual criterion.

Groups 3 and 8 do not differ sufficiently in their EC 50's to meet the requirements of $P = 0.05$, but the difference does satisfy $P = 0.10$. Furthermore, the difference in slopes between Groups 3 and 8 is clearly significant. It would appear therefore, that in Group 8 the small applications of NM used for the sensitivity test may possibly have augmented the sensitivity to MG while, at the same time, increasing the variability of response of the men.

Groups 9 and 10 do not differ significantly either in slope or in EC 50.

Part 4—The Flare Reaction

In the course of a separate series of experiments involving MG burns, cases of "flare reaction" were occasionally observed. By this expression is meant the sudden recrudescence of a healed lesion or an abrupt increase in the severity of a relatively minor lesion after a lengthy period of quiescence. In many of these cases the original lesions had merely been erythemata which had faded and returned apparently to normal only to break out again with severe vesication after a lapse of a week or longer.

An examination of the data on the observed cases indicated that, in every one, the men had been exposed at least twice, and that the flare occurred shortly following a second or later exposure. Although considerable numbers of men had been exposed more than once the number of flares was relatively small. The data were therefore examined in the hope of finding some factor connected with the exposures which was common to, and characteristic of the flare cases.

The only factor which appeared to bear on the subject was the interval between the repeated burns. The examples of flares occurred only in those experiments in which the interval between the burns was about one week or longer. In view of this fact it seemed distinctly probable that the flare reaction was a hypersensitivity phenomenon, and that the significance of the interval between the burns was related to the incubation period required to develop sensitivity. If this were true there were probably other ways in which the hypersensitivity might disclose itself, even when flares did not occur.

It was decided to carry out repeated exposures to MG in an attempt to elicit flare reactions and, at the same time, to measure the skin sensitivity of the men before and after the two burns.

The men used in this study are included in the groups described in the preceding sections. The MG burns were produced by three simultaneous applications of 0.0020 ml. of a 10% (by volume) solution of MG in benzene to one forearm. The second set of burns was produced similarly after an interval of seven days. The two sets of burns were usually, but not invariably, located on the same arm. Variation in this respect did not make any apparent difference in the results.

The lesions were examined daily, and their measurements, together with a visual estimate of their severity, were recorded daily until they were healed.

In a series of 71 men who were given two sets of MG burns in this way, 19 cases of flares involving the sites of the primary lesions were observed. These ranged in severity from simple reactivation, with vesication, of a healed lesion to severe erythema and oedema involving the entire arm, and, in a smaller number of cases, to generalized dermatitis.

It may be remarked in passing that similar data have been obtained with NM. Twenty-four men exposed twice to subvesicant doses of NM at an interval of seven days yielded six cases of flares.

The time of development of the flare varied between one and nine days after the second burn, the average time being four and a half days for the 19 cases of flares induced by MG. In Table IV are given the results of MG sensitivity tests for 18 men who exhibited flare reactions following a second MG burn.

TABLE IV

SENSITIVITY TO MG OF MEN WHO SHOWED FLARE REACTIONS

Log of MG concentration	No. of men tested	% of men reacting
2.097	18	100
1.796	18	89
1.495	18	78
1.194	18	50
0.893	18	33
0.592	18	5

* *Concentration in cu. mm. per liter of solution.*

The calculated EC 50 for this group, in log units, was 1.177 with a standard error 0.059. This figure is significantly less than the EC 50 for Group 3. Indeed this group had the lowest EC 50 (i.e., highest sensitivity) of any of the groups. Thus there appears to be a definite association between the occurrence of the flare reaction and the possession of a high degree of sensitivity to MG.

Discussion

The sensitivity of men to either MG or NM, as measured by the technique illustrated in this paper, is clearly altered by burns induced by MG or NM respectively. Two burns at an interval of a week have a much more marked effect than a single burn, although even a single, small application, such as is involved in a sensitivity test, appears to have some effect after a rest period of about two weeks (Cf. Groups 6 and 7).

No evidence of simple cross sensitivity has been found. Men who had become hypersensitive to MG were still normal toward NM provided that they had not had previous contact with NM. Likewise, men hypersensitive to

NM were normal to MG if they had not had a prior exposure to MG. However, there is some indication that sensitization to MG may be enhanced by simultaneous sensitization to NM (Cf. Groups 3 and 8).

The flare reaction is of particular interest. In our experiments about a quarter of the men who were exposed twice, at an interval of a week, to a small dose of either MG or NM developed the reaction in varying degree. In the MG experiments the initial burns were in all cases quite superficial, indeed in many cases there was merely an erythematous response. The second set of burns was applied at a time when, according to the results of Group 2, there was no general hypersensitivity. Nevertheless this second application, even when made on the opposite arm elicited in certain individuals a second and more severe response at the site of the first lesions. It would appear that although no general sensitivity is present, the actual site of the primary burn is in fact hypersensitive at this time. It was not possible to devise a suitable technique to test this suggestion directly, but the evidence of the flare experiments points strongly in this direction. If it may be assumed that the site of a primary MG lesion becomes hypersensitive after about 11 days—the average time of occurrence of the flares—the possibility suggests itself that such hypersensitivity might be a contributing factor to the notably slow rate of healing of MG burns, particularly in view of the evidence that MG residues are fixed in the skin at the site of application (16).

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